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Journal of Food Composition and Analysis 18 (2005) 139–151

JOURNAL OF FOOD COMPOSITION AND ANALYSIS

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Original Article

A rapid procedure for analysing rice bran tocopherol, tocotrienol and γ-oryzanol contents☆

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Received 4 December 2002; received in revised form 1 July 2003; accepted 12 September 2003

Abstract

Tocopherols, tocotrienols and γ-oryzanol compounds are phytochemicals with antioxidant activities and potential health benefits. Their contents and isomer ratios in rice bran vary among southern US cultivars, suggesting that breeding for higher contents or a favorable ratio of these phytochemicals is feasible. Methods that can rapidly and reliably extract and quantify their contents in rice bran or whole rice kernel are necessary for breeding programs. A simple, one-step equilibrium extraction procedure coupled with reversed-phase (RP) HPLC is presented here. The one-min equilibrium extraction at a 1:60 (w/v) ratio of rice bran to methanol recovered 92 to 102% of the target phytochemicals relative to those of repeated, non-saponified, direct solvent extraction methods. At this 1:60 ratio of bran to solvent, isopropanol and methanol are superior extraction solvents relative to hexane. A modified, mobile-phase gradient with 10% of aqueous phase for the first 3-min eliminates all the methanol-soluble interfering compounds. This extraction method has the following advantages over the currently available methods: speed, no special extraction instrumentation is needed; and the extraction solvent, methanol, is compatible with subsequent quantification via RP-HPLC.
Published by Elsevier Inc.

Keywords: Antioxidants; Phytochemicals; Extraction; Tocopherol; Tocotrienol; γ-Oryzanol; Rice bran

1. Introduction

Antioxidants are thought to protect animal tissues from free radical-mediated degenerative disease and aging. Rice bran contains high levels of several phytochemicals that have antioxidant

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0889-1575/$ - see front matter Published by Elsevier Inc.
doi:10.1016/j.jfca.2003.09.004

activities, as well as other reported health-beneficial properties. Among these phytochemicals, vitamin E, including the four homologs (α, β, γ, and δ) of tocopherols (Ts) and tocotrienols (T3s) (Shin and Godber, 1994), and the γ-oryzanol fraction (Xu and Godber, 1999; Akihisa et al., 2000) have received the most research attention. Historically α-tocopherol has been considered the vitamin-E homolog of greatest value due to its high level of physiological activity, however α-tocotrienol has recently been reported as having more than three times the in vitro free radical scavenging activity of α-tocopherol (Packer, 1995). Tocotrienols also reportedly inhibit cholesterol synthesis, lower serum-cholesterol levels in various animal models, and suppress tumor-cell proliferation, with the γ- and δ-homologs demonstrating greater potency than the α-homolog (Qureshi et al., 2000). Gamma-oryzanol, which is present at 13 to 20 times (w/w) greater content in rice bran than total tocopherols and tocotrienols (Bergman and Xu, 2003), reportedly decreases animal serum-cholesterol levels, has anti-inflammatory activity, and can inhibit cholesterol oxidation in vitro (Rong et al., 1997; Akihisa et al., 2000; Xu et al., 2001). In addition to these putative health benefits, the antioxidants of rice bran and its oil have a potential use as additives to improve the storage stability of foods (Nanua et al., 2000; Kim and Godber, 2001).

Methods reported for the quantification of tocopherols, tocotrienols and γ-oryzanol contents in rice bran involve extraction of rice bran oil (RBO) followed by analysis of the contents of the target antioxidants in the RBO by HPLC. Reported extraction methods include liquid–liquid phase extraction, solid phase extraction, supercritical fluid extraction and the direct solvent extraction (Hu et al., 1996; Shin et al., 1997; Xu and Godber, 2000). Among them, the direct solvent extraction method, which does not require specific extraction instrumentation, has been most commonly used. These methods involve repeated extractions of RBO from the bran, generally with hexane. Under optimal extraction conditions, however, the more polar solvents, isopropanol or isopropanol:hexane (1:1 v/v), extracted more vitamin-E homologs or γ-oryzanol, respectively, from rice bran than did hexane at elevated temperature, even though hexane recovered more RBO (Hu et al., 1996; Xu and Godber, 2000). Methanol, a more polar solvent than isopropanol and hexane, has been used in a direct solvent extraction method for the analysis of oat phenolics, vitamin-E homologs and antioxidant power (Emmons et al., 1999). Information regarding the extraction of γ-oryzanol with methanol, however, is not available, and the comparisons of methanol to hexane and isopropanol for extraction of vitamin-E homologs at ambient temperature need to be made.

Rapid equilibrium extraction methods have been used to extract oil from soy flour, and from rice bran (Sheu, 1987; Clark and Snyder, 1989; Proctor et al., 1994; Proctor and Bowen, 1996). A 1-min hexane extraction at ambient temperature recovered 98% of the oil from soy flour and 90% from rice bran (Clark and Snyder, 1989; Proctor et al., 1994). When isopropanol was used as the extraction solvent in comparison with hexane, the same amount of oil was extracted from the rice bran in 1 min. In a 5-min extraction, however, isopropanol recovered more oil than hexane and 101% relative to that of the AOCS Goldfisch extraction method (Official Methods and Recommended Practices of the American Oil Chemists, 1985; Proctor and Bowen, 1996). In addition, the oil extracted with isopropanol was more stable than hexane-extracted oil to heat-induced oxidation, which suggests that more antioxidants were extracted by isopropanol. This method has not been evaluated for extracting vitamin-E homologs and γ-oryzanol, which are lipophilic antioxidants from rice bran.

With the proposed health benefits and potential industrial uses of the vitamin-E homologs and γ-oryzanol, there is interest in breeding rice cultivars with higher contents or more favorable ratios of these phytochemicals. Breeding rice to have modified levels of phytochemicals will require that studies be performed to quantify the effects of genetics, environment, and post-harvest processing and storage on the levels of these compounds. Various analytical methods for quantifying vitamin E and γ-oryzanol contents are available (Rogers et al., 1993; Hu et al., 1996; Eitenmiller and Landen, 1998). None is suitable for screening the large number of samples required for breeding programs. The objective of this study is to develop and evaluate a rapid and relatively inexpensive procedure for extracting tocopherols, tocotrienols, and γ-oryzanol from rice bran, and to quantify these compounds directly from the extract using reversed-phase (RP)-HPLC.

2. Materials and methods

2.1. Samples

Cypress (CPRS), a conventional US long grain rice variety and Bengal (BNGL), a conventional US medium grain variety were grown in Beaumont, TX using cultural management practices standard for the area. Samples were harvested at approximately 20% moisture, dried to 12% moisture and dehulled using a Satake Rice Huller (Satake, Tokyo, Japan; model THO35A). Immature and diseased kernels were removed by hand.

Fifty grams of each hulled sample were then milled for 30 s using a McGill mill # 1 (HT McGill, Houston, TX) with an 858 g weight in position 12 or 6 for CPRS or BNGL, respectively. The bran (the word bran is used to collectively mean bran and germ) was collected, sieved through a 20-mesh screen, flushed with nitrogen and stored at -20°C.

2.2. Extraction

The solubilities of the target phytochemicals in different solvents were determined using a direct solvent extraction method. Rice bran (50 mg) was extracted in 1-mL solvent: isopropanol (IsOH), IsOH:hexane (1:1) (IH), hexane (Hex) or methanol (MeOH) by vortex mixing vigorously for 1 min at room temperature (RT). A fifth set of samples was extracted with Hex, at 60°C (Hex60), in a water bath for 30 min (mixing every 10 min during incubation) after an initial 15 s of vortex mixing. After extraction, the samples were centrifuged for 10 min at 825*g*. The supernatant was collected and the residue extracted two more times. The solvent in the pooled extract was evaporated and the concentrate dissolved in 3-mL MeOH, filtered (0.45 μm) and analysed by RP-HPLC, as described below. Three extraction replications were used for each solvent experiment.

The MeOH extraction rates for Ts and T3s, and γ-oryzanol from rice bran were determined using an equilibrium extraction method. Rice bran (50 mg of BNGL) was vortex-mixed vigorously in 3-mL MeOH for 1, 3, 6, or 10 min at RT. The mixture was centrifuged for 10 min at 825*g*, filtered, and analysed by RP-HPLC (as described below). For the fifth set of samples, 50 mg of bran was homogenized with a tissue homogenizer (BioSpec Products, Inc., Bartlesville, OK, USA; Tissue-Tearor Model 398) for 30 s at medium speed in 3-mL MeOH; the probe was rinsed two times with 3-mL MeOH each time between samples. The homogenate was centrifuged as above, and the residue extracted two more times by vortex mixing for 1 min with the 3-mL MeOH rinses. The supernatants were pooled and the MeOH evaporated. The concentrate was dissolved in 3-mL MeOH, filtered, and analysed by RP-HPLC, as described below. The recovery rates of the target antioxidants extracted with the equilibrium extraction method were calculated relative to those of the MeOH control, namely the direct MeOH extraction method described above. Four extraction replications were used for each equilibrium experiment and for the homogenization experiment.

2.3. RP-HPLC

The HPLC system consisted of a Waters 2690 Alliance Separations Module, a Waters 2487 Dual Wavelength UV/Vis Absorbance Detector, and a Waters 474 Scanning Fluorescence Detector (Millipore, Bedford, MA). Chromatograms were recorded and processed by Millennium[32] Ver. 3.20 Chromatography Software (Waters). The sample extract was injected through a Sentry guard-column (Waters, Nova-Pak C18, 4 μm, 3.9×20 mm) and separated on a Nova-Pak C18 column (3.9×150 mm, 4 μm, Waters) using a modification of the method of Rogers et al. (1993). The extraction studies were performed using the initial mobile phase conditions of 45% acetonitrile, 45% MeOH, 5% IsOH, and 5% of aq. acetic acid (1%), at a flow rate of 0.8 mL/min, for 6 min. The mobile phase was changed linearly to acetonitrile: MeOH: IsOH at the ratio of 25:70:5 (v/v/v) over the next 10 min and held there for 12 min before being returned to the initial conditions. The Ts and T3 s ($\alpha, \beta + \gamma$, and δ forms) were detected by fluorescence at the excitation and emission wavelengths of 298 and 328 nm, respectively, and the γ-oryzanol by the UV/Vis detector at 325 nm. The chromatographic separations achieved were similar to those reported by Rogers et al. (1993).

An alternative HPLC mobile phase gradient was also developed that is suitable for analysing rice kernels during development, as well as bran from genetically different rice accessions from across the world. The initial mobile phase conditions for this method were 45% acetonitrile, 40% MeOH, 5% IsOH, and 10% of aq. acetic acid (1%) for 3 min, and then changed linearly in the next min to 45% acetonitrile, 45% MeOH, 5% IsOH, and 5% of aq. acetic acid. At 4 min, the mobile phase was changed linearly to acetonitrile:MeOH:IsOH (25:70:5) over the next 10 min and held there for 11 min. The total run time for eluting the Ts, T3s and γ-oryzanol was 25 min. After that, the solvent gradient changed linearly to MeOH: IsOH (95:5) for the next 2 min and held there for 1 min before returning to the initial mobile phase.

2.4. Standards

The standards of Ts were purchased from Matreya, Inc. (Pleasant Gap, PA). Drs. Xu and Godber of Louisiana State University generously provided the standards of the total γ-oryzanol, and the three major components of γ-oryzanol, cycloartenyl ferulate, 24-methylenecycloartanyl ferulate, and campesteryl ferulate. Tocotrienol standards were purified from tocotrienol-rich palm oil concentrate (Life Extension Foundation, Lauderdale, FL) using C18 Sep-Pak cartridges (Waters) and a Symmetry C18 column (4.6×250 mm, 5 μm, Waters). Organic solvent of acetonitrile:methanol (4:1) was added at 1 mL solvent/20 mg of palm oil to extract tocotrienols. The extract was vortex-mixed, sonicated and centrifuged 5 min at 750*g*. A 1-mL aliquot of the upper layer extract was evaporated. The concentrate was dissolved in 100% acetonitrile and

Table 1
Extinction coefficients, maximum wavelengths and quantity ranges of standards

Compound	λ Max(nm)[a]	$E^{1\%}_{1\mathrm{cm}}$	Quantity range of standard curve (ng)
Tocopherol			
α	292	75.8	2.4–49.6
γ	298	91.4	3.0–62.0
δ	298	87.3	0.3–6.2
Tocotrienol			
α	292	91.0	2.4–49.6
γ	296	90.5	3.0–62.0
δ	297	88.1	0.3–6.2
γ-Oryzanol			0.3–6.2

[a] Shin and Godber (1994) and references there in.

loaded onto a C18 Sep-Pak cartridge that was first equilibrated with 100% acetonitrile. The T3s were eluted with 2 mL of 100% acetonitrile. This step eliminated $\geqslant$80% of α-T in the extract. The eluate was separated on the Symmetry C18 column with an initial mobile phase of 75% acetonitrile and 25% methanol for 10 min, and then gradient-increased to 85% methanol in 9 min. The flow rate was 1.2 mL/min. All T3s were eluted before 10 min, and the individual compounds were collected. The α-T was eluted at 18 min. The purity and the stability of the Ts and T3s standards were monitored by the fluorescence detector through RP-HPLC, and by $E^{1\%}_{1\ \mathrm{cm}}$ values (Table 1), measured by a Cary 50 Conc UV–Visible Spectrophotometer (Varian, Victoria, Australia). The quantity range of each standard is listed in Table 1. The response factor of each homolog of T to T3 standard was established and the δ-T, γ-T3 and α-T3 peaks were quantified against δ-T3, γ-T and α-T using their corresponding response factor values. The γ-oryzanol was quantified against the standard curve, which related the known quantity of total γ-oryzanol to the total peak area of the UV absorbance.

2.5. Statistical analysis

All statistical analyses were performed using SAS version 7 (SAS Institute Inc., 1999). Analysis of variance and regression analysis were performed using PROC GLM and PROC REG, respectively. Mean separation analysis was carried out using the Student-Newman-Keuls ($\alpha = 0.05$) test. The percent coefficient of variation (CV, %) was calculated as the standard deviation divided by the arithmetic mean value of the extraction replicates.

3. Results and discussion

The ranges selected for the standard curves were chosen to bracket the ranges found for individual vitamin-E homologs in the bran of US cultivars (Table 1). Each standard exhibited a

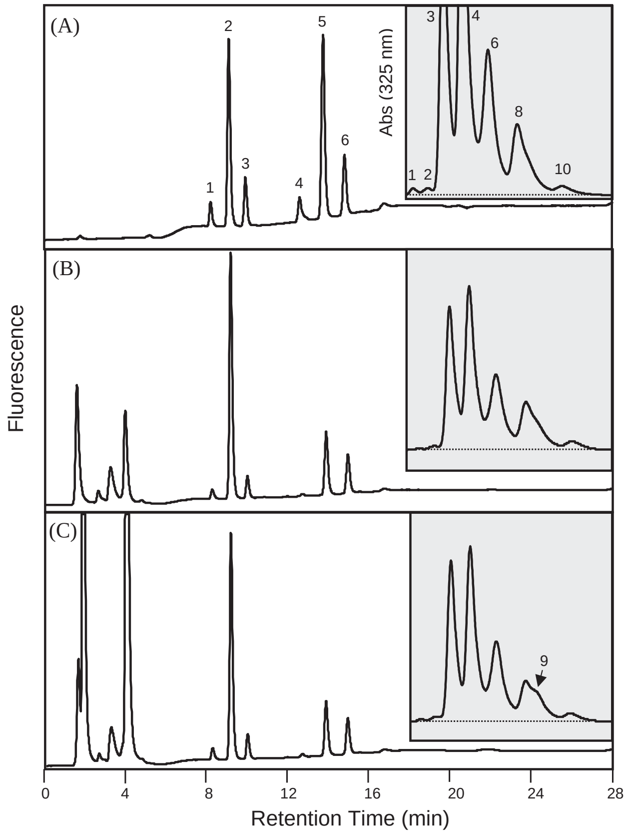

Fig. 1. Chromatograms of the RP-HPLC of the standards of the vitamin-E homologs and total γ-oryzanol, and methanolic extracts of the bran of CPRS, and BNGL. The vitamin-E homologs and γ-oryzanol were detected by fluorescence (Ex 298 nm, Em 328 nm) and UV (325 nm) detectors, respectively. Peaks of vitamin-E homolog standards (A) were: 1, δ-T3 (1.50 ng); 2, γ-T3; 3, α-T3; 4, δ-T (12.02 ng); 5, γ-T (14.99 ng); and 6, α-T. The chromatograms of total γ-oryzanol, the gray inserts, are from retention time ranging from 18 to 24 min. The (A) insert is the standard of total γ-oryzanol, 1.5012 µg; (B) and (C) inserts are total γ-oryzanol from methanolic extracts of the bran of CPRS and BNGL, respectively. Total γ-oryzanol of the bran extracts were quantitated by pooling all the peak areas as shown by the dotted baseline against known standards of total γ-oryzanol calibrated the same way. The peaks were numbered according to Xu and Godber (1999) for ease in identifying the peaks. Peaks 3, 4 and 6, which had the same retention times as the three standard components, were cycloartenyl ferulate, 24-methylenecycloartanyl ferulate, and campesteryl ferulate. Minor peaks 5 and 7 were unresolved, and peak 9 was shown as a shoulder peak in (C) insert.

linear response (of fluorescence for Ts and T3s, and of UV absorbance for the total γ-oryzanol) ($R^2 = 0.99$) over these ranges. Typical chromatograms of standards of Ts, T3s and γ-oryzanol, and of methanolic extracts of bran of CPRS and BNGL are shown in Fig. 1. Baseline separation was achieved for all the Ts and T3s standards except for the coelution of the β- and γ-isomer. The sequence for a typical C18 column elution profile was δ-T3, $(\beta+\gamma)$-T3, α-T3, δ-T, $(\beta+\gamma)$-T and α-T. The response factors of each of the homologs of Ts compared to T3s were established and are as follows: δ-T/δ-T3 = 1.05 ± 0.05 (SD), γ-T/γ-T3 = 1.01 ± 0.02 (SD), and α-T/α-T3 = 1.01 ± 0.05 (SD). The chromatogram of total γ-oryzanol was similar to those of Rogers et al. (1993) and Xu and Godber (1999). The components of γ-oryzanol (Fig. 1A insert) were labeled according to those of Xu and Godber (1999). Peaks 3, 4, and 6, which matched the retention times of the standards of the three major components, were cycloartenyl ferulate, 24-methylenecycloartanyl ferulate, and campesteryl ferulate.

3.1. Direct solvent extraction

Table 2 lists the concentrations of Ts, T3s and γ-oryzanol of CPRS and BNGL obtained by different solvent extractions. The concentration of each compound was converted to % recovery relative to that of the mean concentration value of the MeOH extractions. This allowed evaluation of the recovery of each compound in different solvents to be made across the two varieties. For the vitamin-E homologs, in general, IsOH, IH (1:1) or MeOH extracted more than Hex60, ranging from 2 to 3 times more (Table 2). IsOH and IH extracted more α-homologs, specifically, 12–14% more α-T3 and 8% more α-T, than MeOH. However, there were no significant differences ($P<0.05$) in the contents of δ-T3, $(\beta+\gamma)$-T3, and $(\beta+\gamma)$-T among IsOH, IH and MeOH extracts within each cultivar, except for $(\beta+\gamma)$-T3 of the MeOH-extract of CPRS ($P<0.05$), which was 3.7% less than that of the IsOH-extract. Greater hydrophobicity due to three methyl groups on the chroman ring of the α-homologs compared to the other vitamin homologs might have resulted in their superior solubility in the longer alkyl chain alcohol. The ratios of total T3: total T among the extractions with IsOH, IH and MeOH were similar within each cultivar, with the average of 2.31 for CPRS and 1.71 for BNGL. The concentration of vitamin-E homologs extracted with Hex at ambient temperature had very low reproducibility between days, and the recovery rate was low when compared with Hex60 (data not shown). Therefore ambient Hex extraction results were not used for the comparison among solvents. However, γ-oryzanol was readily extracted by ambient Hex and had 94–96% recovery compared to the Hex60 extractions (data not shown). This suggests that the extractability of the vitamin-E homologs in Hex is more temperature dependent than that of γ-oryzanol. MeOH extracted 10% more γ-oryzanol than Hex60, and the overall recovery (combined CPRS and BNGL) was significantly greater ($P>0.05$). Slightly higher variation, however, in γ-oryzanol for CPRS ($CV = 6.29\%$) resulted in no significant difference being found between the MeOH and Hex60 solvent extractions (Table 2).

The repeatability in extraction of vitamin-E homologs and γ-oryzanol extraction repeatability using each of the solvents was at a level considered acceptable for most analytical purposes ($CV \leqslant 5.3\%$), except for a few compounds extracted by Hex60 (Table 2). The repeatability across cultivars was demonstrated by the CV (%) of the mean recovery for the two cultivars (<4.5% for IsOH, IH, and MeOH). The Hex60 had a high CV for the mean recovery of α-vitamin E homologs due to a 55% recovery from the CPRS bran and 40% from the BNGL relative to the recoveries from MeOH.

Hu et al. (1996) demonstrated that increasing the solvent to bran ratio from 2:1 to 3:1 IsOH resulted in the recovery of 10% more vitamin E homologs and 8.4% more γ-oryzanol from crude bran oil. Under their optimal extraction conditions (3:1 solvent to bran ratio and 60°C), IsOH extracted more vitamin-E homologs and a similar amount of γ-oryzanol relative to hexane (Hu et al., 1996). We have demonstrated here that when using ambient-temperature direct solvent extraction, repeated for three times, and with a solvent to bran ratio of 20:1 (v/w), both IsOH and MeOH recovered more than 2.3 times the amount of total vitamin-E homologs compared to extractions using Hex at 60°C. Proctor and Bowen (1996), using an ambient-temperature

Table 2
Comparison of concentrations and recoveries of antioxidants in rice bran using direct solvent extraction method[a]

Solvent[b]		δ-T3			β,γ-T3			α-T3			β,γ-T			α-T			γ-Oryzanol		
		CPRS	BNGL	Mean	CPRS	BNGL	Mean	CPRS	BNGL	Mean	CPRS	BNGL	Mean	CPRS	BNGL	Mean	CPRS	BNGL	Mean
IsOH	Mean[c]	4.6a	6.2a		163.5a	127.5a		54.8a	54.5a		24.2a	29.4a		72.0a	80.9a		3.7a	4.1a	
	CV, %	3.6	1.4		0.9	1.6		2.2	2.0		1.3	0.7		2.0	1.3		3.1	1.9	
	Recovery, %	105.0	99.2	102.1a	103.7	103.0	103.3a	114.6	113.9	114.2a	101.9	99.7	100.8a	109.1	108.7	108.9a	94.9	98.3	96.6ab
	CV of mean recovery, %			4.0			1.2			1.9			1.5			1.5			3.0
IH	Mean[c]	4.6a	6.1a		164.0a	124.7a		55.0a	52.6a		24.6a	29.6a		73.7a	78.5ab		3.6a	4.1a	
	CV, %	1.9	1.6		0.4	1.0		0.8	0.8		1.1	1.8		0.4	3.7		5.2	1.4	
	Recovery, %	103.9	97.6	100.7a	104.0	100.7	102.4ab	115.1	112.5	112.5a	103.2	100.2	101.7a	111.7	105.5	108.6a	91.9	97.2	94.6bc
	CV of mean Recovery, %			3.8			1.9			2.6			2.1			3.9			4.5
MeOH	Mean[c]	4.4a	6.2a		157.7b	123.9a		47.8b	47.8b		23.8a	29.5a		66.0b	74.4b		3.9a	4.2a	
	CV, %	3.7	0.6		2.2	2.5		2.9	3.8		1.6	2.8		2.6	5.3		4.3	2.0	
	Recovery, %	100.0	100.0	100.0a	100.0	100.0	100.0b	100.0	100.0	100.0b	100.0	100.0	100.0a	100.0	100.0	100.0b	100.0	100.0	100.0ab
	CV of mean recovery, %			2.3			2.1			3.1			2.0			3.7			3.0
Hex60	Mean[c]	3.4b	4.5b		53.2c	43.5b		26.7c	19.2c		9.8b	12.4b		37.1c	29.4c		3.4a	3.8b	
	CV, %	2.7	5.5		1.4	11.4		4.8	4.7		2.7	10.8		6.6	6.4		6.3	3.1	
	Recovery, %	76.3	71.7	74.0b	33.7	35.1	34.4c	55.9	40.1	48.0c	41.2	41.9	41.6b	56.2	39.5	47.9c	87.4	91.4	89.4c
	CV of mean recovery, %			5.1			7.8			18.5			7.2			20.1			5.0

[a] Different letters within the column indicate the means are significantly different ($P<0.05$).
[b] Solvent abbreviations of IsOH, IH, MeOH and Hex60 are isopropanol, isopropanol: hexane (1:1), methanol and hexane at 60°C, respectively.
[c] µg/g bran fresh weight for δ-T3, β,γ-T3, α-T3, β,γ-T and α-T; mg/g bran fresh weight for γ-oryzanol.

equilibrium procedure, extracted rice bran oil with IsOH and Hex. These authors noted that the Hex-extracted oil after storage at elevated temperature showed a classical oxidation curve and increased in weight due to peroxide formation. The IsOH-extracted oil showed little weight change during the incubation period. The authors suggested that the greater amount of vitamin-E homolog extracted by IsOH compared to Hex might have contributed to the oil's oxidative stability. Our data supports their hypothesis. The hydroxyl groups on the chroman rings of vitamin-E homologs and on the benzene ring of ferulate esters might make these compounds more extractable in alcohol than in hexane. Our results demonstrate that IsOH and MeOH are suitable for extracting vitamin-E homologs and γ-oryzanol from rice bran at ambient temperature.

The concentration of total vitamin-E homologs and the ratio of the individual compounds in the bran as obtained from MeOH or IsOH extraction closely match the values reported by Shin et al. (1997), in which the rice bran was saponified and then extracted either by liquid–liquid or solid-phase extraction. The Hex60 extract gives similar concentration values of the total vitamin-E homologs in the bran compared to those reported by Hu et al. (1996). In their study the direct solvent extraction method was used, and the solvents were IsOH or Hex at the solvent to bran ratio (v/w) of 3:1.

The γ-oryzanol concentration measured here ranged from 3.4 to 3.9 mg/g bran fresh wt of CPRS and 3.8 to 4.2 mg/g for BNGL. The % moisture of rice bran upon similar lab preparation is in average around 8% (personal observation). The γ-oryzanol values are comparable to the 3.6–5.4 mg/g dried rice bran reported by Bergman and Xu (2003), the 4.74 mg/g of US long grain and the 4.0 mg/g of Bengal from composite dried rice bran reported by Lloyd et al. (2000), and the 3.4 mg ester/g of dried rice bran reported by Norton (1995).

3.2. Equilibrium extraction

Table 3 lists the results from the equilibrium extraction of Ts, T3s and γ-oryzanol using MeOH for 1, 3, 6, and 10 min, compared with the MeOH control and homogenized sample. There was no significant difference ($\alpha = 0.05$) in the recovery of δ-T3, $(\beta+\gamma)$-T3, δ-T and $(\beta+\gamma)$-T among the MeOH control and the equilibrium extractions of 1, 3, 6, and 10 min. For α-homologs, the equilibrium extraction for 1 min allowed recovery of these compounds comparable to those of MeOH control. Increasing extraction time, however, decreases the recovery of α-T3 and α-T. The 10-min extraction recovered only 78% of α-T3 and 82% of α-T relative to the MeOH control, and the homogenized extract had the lowest recovery. For γ-oryzanol, a 1-min extraction recovered 95% of that obtained with the MeOH control. Increasing the extraction time to 10 min only increased the recovery by 1.5%. The data suggests that after 1 min the concentrations of the antioxidants reached equilibrium both inside and outside of the bran. Thus, the rice bran was a fine enough particle size, or sufficient cellular disruption resulted from the milling process, such that no further homogenization of rice bran during extraction is necessary.

The equilibrium extraction method has previously been used to extract oil from soy flour and from rice bran (Sheu, 1987; Clark and Snyder, 1989; Proctor et al., 1994; Proctor and Bowen, 1996). At ambient temperature for 1 min, Hex extracted 98% and 90% of the oil from soy flour and rice bran, respectively (Clark and Snyder, 1989; Proctor et al., 1994) compared with the AOCS Goldfisch extraction method. When IsOH was used as the extraction solvent in comparison with Hex, the same amount of RBO is extracted during 1 min. In 5 min, IsOH

Table 3
Comparison of concentrations and recoveries of antioxidants from BNGL rice bran among the extraction methods using MeOH as solvent

Extraction method		Vitamin-E homologs[a] (μg/g bran)						γ-Oryzanol[a](mg/g bran)
		δ-T3	β,γ-T3	α-T3	δ-T	β,γ-T3	α-T	γ-Oryzanol
Direct Solvent	Mean	6.22a	123.86a	47.83a	2.34a	29.53a	74.40a	4.21a
Control	CV, %	0.60	2.49	3.85	5.29	2.76	5.27	2.00
	Recovery, %	100.0	100.0	100.0	100.0	100.0	100.0	100.0
Equilibrium	Mean	6.24a	126.76a	44.21a	2.33a	29.00a	69.22a	3.99b
1 min	CV, %	3.40	1.50	8.44	2.23	4.08	3.16	0.71
	Recovery, %	100.3	102.3	92.4	99.6	98.2	93.0	94.8
Equilibrium	Mean	6.03a	125.91a	42.12ab	2.26a	27.49a	63.14b	4.02b
3 min	CV, %	1.62	1.62	7.37	6.63	3.57	2.15	1.49
	Recovery, %	97.0	101.7	88.1	96.7	93.1	84.9	95.5
Equilibrium	Mean	6.07a	124.99a	36.56bc	2.23a	27.53a	61.28b	4.03ab
6 min	CV, %	0.92	0.56	5.23	5.22	2.91	3.46	0.87
	Recovery, %	97.7	100.0	76.4	95.3	93.2	82.4	95.9
Equilibrium	Mean	6.13a	125.10a	37.13bc	2.34a	28.44a	60.95b	4.06ab
10 min	CV, %	1.52	1.46	5.99	6.26	4.26	5.34	2.16
	Recovery	98.6	101.0	77.6	99.9	96.3	81.9	96.4
Homogenization	Mean	5.51b	112.86b	35.40c	2.29a	28.54a	58.04b	3.46c
	CV, %	1.96	3.05	2.87	7.61	3.70	5.79	4.10
	Recovery	88.6	91.1	74.0	97.8	96.7	78.0	82.3

[a] Different letters within the column indicate the means are significantly different ($P<0.05$).

recovered more RBO than Hex and recovered 101% oil relative to the AOCS Goldfisch extraction method. The solvent to bran ratio in these studies was 10:1 (Proctor et al., 1994; Proctor and Bowen, 1996). The equilibrium extraction method is used here for extracting lipophilic phytochemicals in rice bran with MeOH, at a solvent to bran ratio of 60:1. With this condition, a 1-min extraction is sufficient to recover 92–102% of the target compounds, relative to the control. Our results indicate that this rapid equilibrium extraction recovered similar amounts of the targeted compounds compared to the repeated, direct-solvent extraction method.

Using equilibrium extraction with methanol as the extraction solvent has several advantages. Firstly, the same MeOH extract of rice bran is suitable for studying various phytochemical related traits such as antioxidant activity, and the total and individual phenolics, vitamin-E homologs and γ-oryzanol. Secondly, the resulting extract can be used directly for RP-HPLC analysis, while requiring only a small amount of rice bran sample (<50 mg of rice bran or whole grain rice flour) and a quick sample preparation. The use of reverse-phase, instead of normal-phase HPLC, under the gradient mobile phase used here, allows separation of γ-oryzanol into seven fractions, especially the three major components, cycloartenyl ferulate, 24-methylenecycloartanyl ferulate, and campesteryl ferulate (peaks 3, 4, and 6 in Fig 1 insert). The minor peaks of 5, 7, and 9 were not resolved. However, Xu and Godber (1999) were able to resolve all 10 components of γ-oryzanol using reverse-phase HPLC. The normal-phase HPLC can only separate γ-oryzanol into two fractions (Dlack and Saska, 1994). Since these three components have different specific antioxidant activities against cholesterol oxidation (Xu et al., 2001), the concentrations of these individual components within each cultivar provide useful information for breeding purposes. Nevertheless, these three major peaks were not resolved to the baseline. Quantification for each of the three major peaks was not performed here because the co-eluted minor components could not be positively identified without available standards.

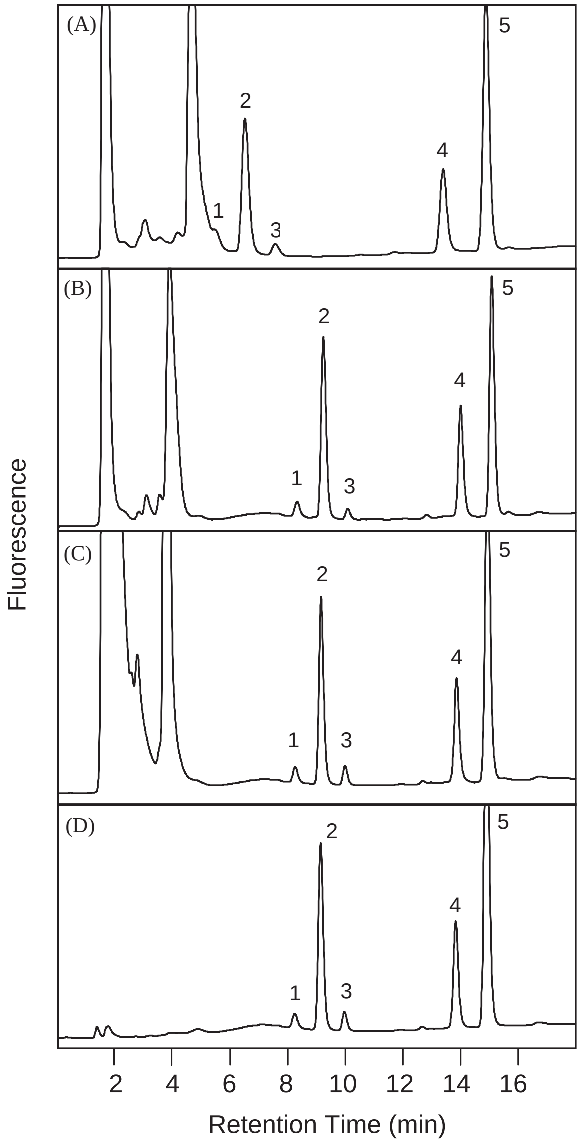

Fig. 2. Chromatograms of the RP-HPLC of the vitamin-E homologs of rice flour extracts using fluorescence detection (Ex 298 nm, Em 328 nm). The immature, whole rice kernel flour of CPRS was extracted with IsOH (A, B) or MeOH (C) at room temperature, and with Hexane at 60°C (D) using the direct solvent extraction method. Two mobile phase gradient programs, the original (A) and the modified (B, C, D), were used (described in Section 2). Peak 1, δ-tocotrienol; peak 2, β,γ-tocotrienols; peak 3, α-tocotrienol; peak 4, β,γ-tocopherols; peak 5, α-tocopherol. The peak heights or peak areas are not comparable among chromatograms because the injection volume and the concentrations of the extracts were different.

3.3. Mobile phase

The MeOH-extract or IsOH-extract of bran from several accessions in a rice germplasm collection and from rice kernels during development, including hull, had large quantities of compounds that eluted just before δ-T3 and fluoresced at the same wavelength as the vitamin-E homologs (unpublished results). These compounds made accurate quantification of δ-T3 difficult. By increasing the % aqueous solution of the initial mobile phase from 5% to 10% for the first 3 min, all of the interfering compounds eluted earlier and the elution of δ-T3 was delayed (Fig. 2). The results obtained from the IsOH-extract of immature whole kernels of CPRS using the original mobile-phase gradient are displayed in Fig. 2A. There is a large peak that elutes before δ-T3. The initial mobile phase of 10% aq. acetic acid for 3 min separates the interfering substances from δ-T3, and accurate quantification of δ-T3 can be achieved (Fig. 2B and C). These interfering compounds are more hydrophilic and are not soluble in Hex (Fig. 2D). This new mobile-phase gradient has been proven applicable for allowing analysis of the methanol-extract of rice bran from over 200 genetically different rice accessions collected across the world, and of whole rice kernels through the course of seed maturation (unpublished results). This new mobile-phase gradient system can be applied to a wide range of samples for the analysis of vitamin-E homologs and γ-oryzanol when samples are extracted with IsOH or MeOH that are relatively polar, and when the extracts are analysed on RP-HPLC.

4. Conclusion

The described rapid equilibrium extraction method using MeOH as a solvent coupled with RP-HPLC provides a fast, relatively inexpensive, reproducible extraction procedure for assaying rice bran Ts, T3s and γ-oryzanol components. Also, the new HPLC mobile-phase gradient reported will enable the analysis of vitamin-E homologs and γ-oryzanol from noncommercial rice bran samples that have been extracted with relatively polar solvents. This method will facilitate breeding efforts focused on enhancing rice bran phytochemical contents, as well as physiology studies designed to understand the plant metabolism of vitamin-E homologs and γ-oryzanol.

Acknowledgements

The authors wish to thank Z. Xu and J.S. Godber of Louisiana State University for their generosity providing the standards, and for their discussion on extraction and quantification methods. The excellent technical assistance of Janis Delgado is greatly appreciated.

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